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Deletion of the Three Distal S1 Motifs of *Saccharomyces cerevisiae* Rrp5p Abolishes Pre-rRNA Processing at Site A_2 without Reducing the Production of Functional 40S Subunits

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Yeast Rrp5p, one of the few *trans*-acting proteins required for the biogenesis of both ribosomal subunits, has a remarkable two-domain structure. Its C-terminal region consists of seven tetratricopeptide motifs, several of which are crucial for cleavages at sites A_0 to A_2 and thus for the formation of 18S rRNA. The N-terminal region, on the other hand, contains 12 S1 RNA-binding motifs, most of which are required for processing at site A_3 and thus for the production of the short form of 5.8S rRNA. Yeast cells expressing a mutant Rrp5p protein that lacks S1 motifs 10 to 12 (mutant *rrp5Δ6*) have a normal growth rate and wild-type steady-state levels of the mature rRNA species, suggesting that these motifs are irrelevant for ribosome biogenesis. Here we show that, nevertheless, in the *rrp5Δ6* mutant, pre-rRNA processing follows an alternative pathway that does not include the cleavage of 32S pre-rRNA at site A_2. Instead, the 32S precursor is processed directly at site A_3, producing exclusively 21S rather than 20S pre-rRNA. This is the first evidence that the 21S precursor, which was observed previously only in cells showing a substantial growth defect or as a minor species in addition to the normal 20S precursor, is an efficient substrate for 18S rRNA synthesis. Maturation of the 21S precursor occurs via the same endonucleolytic cleavage at site D as that used for 20S pre-rRNA maturation. The resulting D-A_3 fragment, however, is degraded by both 5′→3′ and 3′→5′ exonuclease digestions, the latter involving the exosome, in contrast to the exclusively 5′→3′ exonucleolytic digestion of the D-A_2 fragment. We also show that *rrp5Δ6* cells are hypersensitive to both hygromycin B and cycloheximide, suggesting that, despite their wild-type growth rate, their preribosomes or ribosomes may be structurally abnormal.

Ribosome biogenesis in eukaryotes largely takes place in the nucleolus, a specialized compartment of the nucleus, and proceeds via a complex series of steps, including transcription, modification, and processing of the pre-rRNA, integrated with the ordered assembly of various precursor species with ribosomal proteins.

Pre-rRNA transcription involves multiple copies of two types of transcriptional units, one encoding 5S rRNA and the other containing the genes for 18S, 5.8S, and 25S/28S rRNAs. The latter species therefore are transcribed as a single large precursor, which contains external transcribed spacers at either end (5′-ETS and 3′-ETS) as well as two internal transcribed spacers (ITS1 and ITS2) separating the mature rRNA sequences (Fig. 1A). This primary transcript is modified extensively by ribose methylation as well as pseudouridylation (14, 15), followed by removal of the spacers in a series of endonucleolytic and exonucleolytic processing reactions.

Eukaryotic pre-rRNA maturation has been characterized in most detail in the yeast *Saccharomyces cerevisiae* (reviewed in references 20, 28, 38, and 52) (Fig. 1B), where the first detectable pre-rRNA molecule is the 35S species, generated through endonucleolytic cleavage by Rnt1p in the 3′-ETS shortly downstream from the 3′ end of mature 25S rRNA (1, 29). Cleavages at sites A_0, A_1, and A_2 then lead to the formation of the 20S and 27S A_2 ($27SA_2$) pre-rRNAs. Endonucleolytic cleavage at site D, which occurs in the cytoplasm in yeast cells but not in other eukaryotic cells, removes the remaining ITS1 sequences from 20S pre-rRNA to form the 3′ end of mature 18S rRNA (36, 40, 49).

The $27SA_2$ pre-rRNA is processed into two forms of 27SB pre-rRNA via different pathways. The major one involves endonucleolytic cleavage at site A_3 by RNase MRP, resulting in $27SA_3$ pre-rRNA (13, 31, 32, 39); the latter is then rapidly converted into 27SB short ($27SB_S$) pre-rRNA by 5′→3′ exonucleolytic digestion to site $B1_S$, the 5′ end of short-form mature 5.8S rRNA (25). The minor pathway involves purported endonucleolytic cleavage at site $B1_L$, located 6 or 7 nucleotides (nt) upstream of $B1_S$, to give 27SB long ($27SB_L$) pre-rRNA having the 5′ end of long-form mature 5.8S rRNA (38, 52). Both 27SB species are cleaved at site C_2 within ITS2 to give the 7S and 25.5S precursors, from which the remaining ITS2 sequences are removed exonucleolytically (19, 22, 33, 34, 48).

In addition to the ribosomal proteins, a large number of nonribosomal factors play a crucial role in eukaryotic ribosome biogenesis. These *trans*-acting factors include numerous small nucleolar ribonucleoproteins as well as a host of non-small nucleolar ribonucleoproteins. With very few exceptions, inactivation of a specific *trans*-acting factor affects the biogenesis of only one of the ribosomal subunits and not the other (20, 28, 38, 52). One such exception is nucleolar protein Rrp5p, which was shown to be involved in the formation of both subunits (18, 45, 51); another is the recently identified protein Rrp12p (37).

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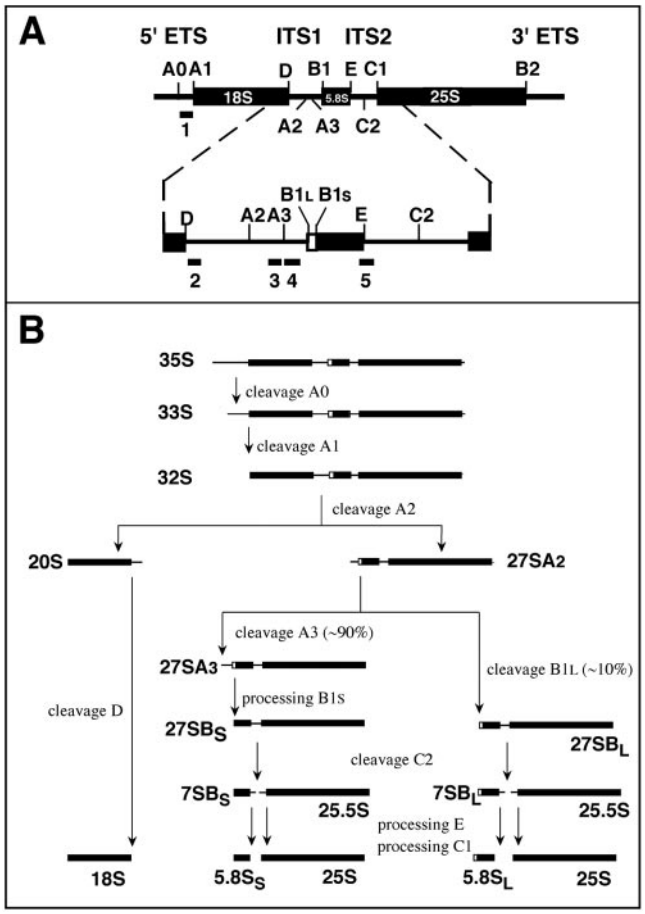

FIG. 1. Processing of pre-rRNA in *S. cerevisiae*. (A) Structure of rDNA transcription unit. Thick bars represent the mature 18S, 5.8S, and 25S rRNA sequences, and thin bars represent the spacer sequences. Processing sites and locations of oligonucleotides used in this study are depicted. ETS, external transcribed spacer. (B) pre-rRNA processing pathway.

Rrp5p is an essential 193-kDa protein having a remarkable two-domain structure (18, 45) (Fig. 2). The N-terminal two thirds of the protein contain 12 S1 RNA-binding motifs, while the C-terminal portion consists of seven tetratricopeptide (TPR) repeats, motifs thought to be involved in protein-protein interactions (9, 30). Mutational analysis indicated that the two domains are also functionally distinct; deletion of various combinations of the first eight S1 RNA-binding motifs of the protein blocked processing at site A_3 and shifted the production of 5.8S rRNA from the short form to the long form as the predominant species (17, 18). Moreover, in the deletion mutants, a new processing site in ITS1, named A_4, was found midway between sites A_2 and A_3 (17). Deletion mutations in several of the TPR motifs, on the other hand, inhibited the early cleavage steps at sites A_0 to A_2, resulting in the depletion of 20S pre-rRNA and thus 18S rRNA (18, 45).

Yeast cells dependent upon mutant Rrp5p that lacks S1 RNA-binding motifs 10 to 12 (amino acids 1132 to 1354) grow normally and produce wild-type levels of all mature rRNAs (18), suggesting that S1 motifs 10 to 12 are irrelevant for ribosome biogenesis. However, detailed analysis of pre-rRNA processing was not carried out with this *rrp5Δ6* mutant to exclude the possibility of a deviation that would not affect its ultimate outcome. The data reported in this article demonstrate that such a deviation does indeed occur in mutant cells. The absence of S1 motifs 10 to 12 leads to an Rrp5p protein that no longer supports cleavage at site A_2. Consequently, in mutant cells, the 32S precursor is cleaved directly at site A_3, giving rise to a 3′-extended 21S intermediate rather than the normal 20S pre-rRNA. The 21S pre-rRNA is then converted into mature 18S rRNA by normal cleavage at site D. The use of this alternative pathway does not significantly affect the levels of mature rRNAs or the rates of growth of the cells, demonstrating that 21S pre-rRNA is an efficient substrate for 18S rRNA synthesis. However, mutant cells are more sensitive to several antibiotics, possibly reflecting a structural abnormality in their ribosomes.

MATERIALS AND METHODS

Plasmids and strains. *Escherichia coli* strain MH1 was used for the cloning and propagation of plasmids. Yeast strains used in this study are listed in Table 1. Plasmids pTRP1-*ProtA*::*rrp5* and pTRP1-*ProtA*::*rrp5Δ6* were described by Eppens et al. (18). Mutant ribosomal DNA (rDNA) plasmids pT and pTM3 were described by Van Beekvelt et al. (46). Yeast transformation was performed as described by Gietz et al. (24). Strains YJV163 and YJV207 were obtained by transformation of strain YJV148 (*rrp5*::*HIS3* pURA-*RRP5*) with pTRP1-*ProtA*::*rrp5* and pTRP1-*ProtA*::*rrp5Δ6*, respectively (18). Transformants were grown on selective plates containing 2% glucose and then transferred to plates containing 5-fluoroorotic acid to select against plasmid pURA-*RRP5* and thus effect the plasmid shuffle (10). Positive colonies were checked for uracil auxotrophy.

For the integration of the *ProtA*::*rrp5Δ6* allele, we first cloned the 5.8-kb XbaI-PstI fragment from plasmid pTRP1-*ProtA*::*rrp5Δ6* into pUC19. The *LEU2* marker from pYDp-L (8) was cloned into the SacII site of the upstream sequence of the *RRP5* promoter of the plasmid-encoded mutant allele. The resulting plasmid was used to create a linear XbaI-PstI integration fragment, which was then transformed into strains YJV140 and YCA20 (the latter a kind gift from D. Tollervey). Leu^+ prototrophic colonies were selected and screened by PCR to check for correct replacement of the genomic wild-type *RRP5* gene by the *rrp5Δ6* allele, resulting in strains YHV124 and YCA20Δ6. Proper expression of the mutant protein in these strains was checked by Western analysis.

Antibiotic sensitivity assay. Freshly grown colonies of YJV163 and YJV207 were resuspended in sterile water and 10-fold serial dilutions were spotted onto yeast extract-peptone-dextrose (YPD) plates containing no antibiotics, 400 μg of paromomycin/ml, 50 μg of hygromycin B/ml, or 200 μg of cycloheximide/ml. Plates were incubated at 30°C for 3 days.

RNA analysis. Cells were grown in the appropriate selective medium lacking methionine but containing either 2% glucose or 1% galactose–1% raffinose–1% sucrose (GRS) as the carbon source. To inhibit 5′→3′ exonucleases, we incubated cells with 0.2 M LiCl for 1 or 2 h before harvesting the cultures. Cells were harvested at approximately 20 units of optical density at 600 nm. Isolation of RNA, Northern hybridization, and primer extension analysis were carried out as described previously (53). Oligonucleotides used as probes in these analyses are listed in Table 2, and their locations are depicted in Fig. 1A.

RESULTS

The *rrp5Δ6* deletion mutant shows antibiotic hypersensitivity. To investigate whether there is a previously unreported role for S1 motifs 10 to 12 in ribosome biogenesis, we decided first to test *rrp5Δ6* mutant cells for possible antibiotic sensitivity. Several reports have shown that yeast strains defective in the synthesis of either the large or the small ribosomal subunit are hypersensitive to the aminoglycoside antibiotic paromomycin (26, 27, 54). Furthermore, hygromycin B sensitivity has been reported for several mutants that are impaired in normal 60S subunit production, while cycloheximide sensitivity has

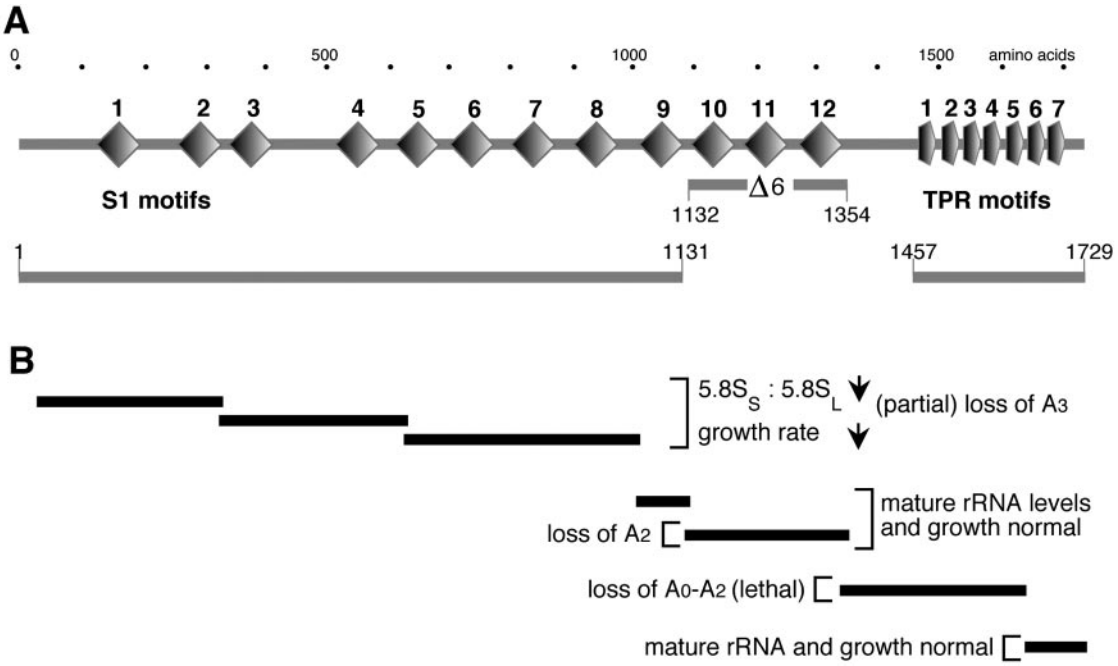

FIG. 2. Structure and function of Rrp5p. (A) The positions of the S1 RNA-binding motifs and the TPR motifs are indicated by triangles and pentagrams, respectively. The region removed by the Δ6 mutation and the fragments expressed in the bipartite *rrp5N2-rrp5C1* mutant (43) (see Discussion) are shown below the structure. (B) Previously constructed deletion mutants and their processing and growth phenotypes (18).

also been used as an indicator for altered 60S subunit maturation (7, 26, 35).

Using the *rrp5*-null yeast strain YJV148 (Table 1), we replaced its *URA3* plasmid containing the wild-type *RRP5* gene with a *TRP1* plasmid encoding the *rrp5Δ6* deletion mutant and spotted the resulting strain, together with a control expressing the wild-type gene, in 10-fold serial dilutions on YPD plates containing paromomycin (400 μg/ml), hygromycin B (50 μg/ml), or cycloheximide (200 μg/ml). At these concentrations, the wild-type cells showed no or at most only a very limited reduction in growth (Fig. 3, upper rows). Growth of the mutant cells, however, was significantly retarded by all three antibiotics (Fig. 3, lower rows), the strongest effects being seen for hygromycin B (Fig. 3C) and cycloheximide (Fig. 3D). These results suggest that the *rrp5Δ6* mutation does affect ribosome biogenesis and prompted us to analyze the pre-rRNA processing phenotype of this mutant in more detail.

S1 RNA-binding motifs 10 to 12 are essential for the processing of 32S pre-rRNA at site A_2. To monitor pre-rRNA processing in the *rrp5Δ6* mutant, we performed Northern analysis of total RNA isolated from exponentially growing cells that depend on either Rrp5Δ6p or wild-type Rrp5p. As observed previously (18), the *rrp5Δ6* strain accumulates mature 18S and 25S rRNAs to wild-type levels (Fig. 4A). However, analysis of the various precursor rRNA species demonstrated that pre-rRNA processing in the mutant strain did differ from that in its wild-type counterpart. We were unable to detect $27SA_2$ pre-rRNA in the *rrp5Δ6* cells by using probe 3, which is complementary to the pre-rRNA just upstream from site A_3 (Fig. 4B). Instead, this probe visualized a product that migrated slightly more slowly than the 20S pre-rRNA (Fig. 4B, lane 6). This result can be seen more clearly by comparing lanes 3 and 4 of Fig. 4B, where hybridization was carried out with probe 2, which is located shortly downstream from site D. This probe detected the 20S rRNA band in the wild-type cells (Fig. 4B, lane 3) and the slightly longer product in the mutant cells (lane 4). Since the latter band was not visualized by probe 1, located just upstream from site A_1 (Fig. 4B, lane 2), or probe 4, which

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
YJV140	*MAT***a** *ade2 his3 leu2 trp1 ura3*	51
YJV148	*MAT***a** *ade2 his3 leu2 trp1 ura3 rrp5*::*HIS3* pURA3-*RRP5*	18
YJV163	*MAT***a** *ade2 his3 leu2 trp1 ura3 rrp5*::*HIS3* pTRP1-*ProtA*::*rrp5*	18
YJV207	*MAT***a** *ade2 his3 leu2 trp1 ura3 rrp5*::*HIS3* pTRP1-*ProtA*::*rrp5Δ6*	This study
YHV124	*MAT***a** *ade2 his3 leu2 trp1 ura3 ProtA*::*rrp5Δ6* (*LEU2*)	This study
YHV134	YHV124 + pT	This study
YHV136	YHV124 + pTM3	This study
YCA20	*MAT***a** *his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108 GAL10*::*RRP45 RRP5*	5
YCA20Δ6	*MAT***a** *his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108 GAL10*::*RRP45 ProtA*::*rrp5Δ6* (*LEU2*)	This study

TABLE 2. Oligonucleotides used as probes in this study

Probe	Nucleotide sequence
1	5′-TAAAAGAAGAAGCAACAAGCAG-3′
2	5′-GCTCTCATGCTCTTGCCAAAAC-3′
3	5′-TGTTACCTCTGGGCCC-3′
4	5′-CCAGTTACGAAAATTCTTGTTTTTGAC-3′
5	5′-GAATGTTTGAGAAGGAAATGACGCTC-3′

is complementary to the pre-rRNA sequence between sites A_3 and B_1 (lane 8), its 5′ and 3′ ends probably coincide with sites A_1 and A_3, respectively. Together, these results indicate that in the *rrp5Δ6* mutant, processing at site A_2 is strongly impaired and the 32S pre-rRNA is instead cleaved directly at site A_3. This alternative processing route appears to have somewhat slower kinetics than the normal one, as concluded from the increased level of 32S pre-rRNA in the mutant cells (Fig. 4B). No $27SA_3$ precursor was detected by probe 4 (Fig. 4B, lane 8), because this precursor is rapidly converted into the $27SB_S$ species and is barely detectable even in wild-type cells (25) (Fig. 5, lane 1).

In order to document the inhibition of 32S pre-rRNA cleavage at site A_2 more quantitatively, we performed a reverse transcription assay with probe 5, which is complementary to the sequence that spans the 3′ end of 5.8S rRNA and thus hybridizes to the 27SA and 27SB as well as the 7S precursor species. A prominent stop corresponding to site A_2 was visible when RNA from the wild-type strain was used as the template (Fig. 5, lane 1), but there was no detectable signal at this position upon analysis of RNA from the mutant strain (lane 2). Signals corresponding to site A_3 were generated with RNA from the wild-type and mutant cells, although in the latter the level of the $27SA_3$ precursor seemed to be somewhat lower, indicating an even more rapid conversion into $27SB_S$ pre-rRNA in the mutant cells than in the wild-type cells. In summary, therefore, the data demonstrate a crucial role for the region of Rrp5p that encompasses S1 motifs 10 to 12 in the processing of 32S pre-rRNA at site A_2.

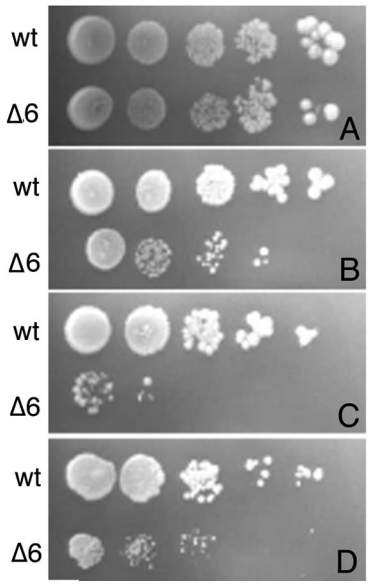

FIG. 3. Comparison of antibiotic sensitivities of strains YJV163 (*rrp5*::*HIS3* pTrp-*ProtA*::*RRP5*; indicated as wt) and YJV207 (*rrp5*::*HIS3* pTrp-*ProtA*::*rrp5Δ6*; indicated as Δ6). Ten-fold serial dilutions of equal numbers of freshly grown cells of the two strains were spotted onto YPD plates containing no antibiotic, paromomycin (400 μg/ml), hygromycin B (50 μg/ml), or cycloheximide (200 μg/ml) and incubated at 30°C for 3 days.

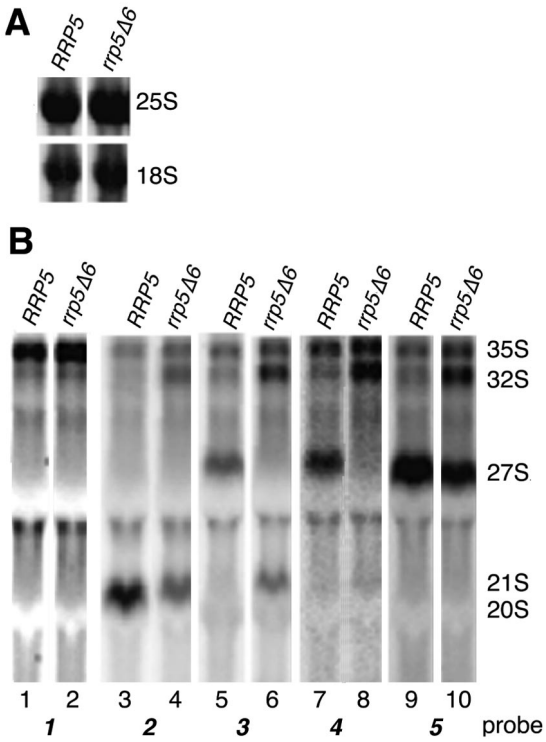

FIG. 4. Effect of the *rrp5Δ6* mutation on pre-rRNA processing. Total RNA was isolated from exponentially growing YJV163 or YJV207 cells expressing ProtA-Rrp5p (lanes 1, 3, 5, 7, and 9) or ProtA-Rrp5Δ6p (lanes 2, 4, 6, 8, and 10), respectively. RNA was separated on a 1.2% agarose gel containing formaldehyde and subjected to Northern analysis with the indicated probes. (A) Ethidium bromide staining to visualize mature 18S and 25S rRNAs. (B) Northern analysis. Figure 1A shows the locations of the probes. The band labeled 27S corresponds to $27SA_2$ pre-rRNA for probe 3, to $27SA_2$ and $27SA_3$ pre-rRNAs for probe 4, and to $27SA_2$, $27SA_3$, $27SB_S$, and $27SB_L$ pre-rRNAs for probe 5.

The 21S pre-rRNA species was observed previously in mutant yeast strains in which cleavage at site A_2 was inhibited by inactivation of the genes encoding ribosomal proteins Rps0p and Rps21p (41), by depletion of various *trans*-acting factors, including Rrp7p (6) and Rrp8p (12), or by expression of two noncontiguous fragments of Rrp5p, consisting of S1 motifs 1 to 9 and TPR motifs 1 to 7 (43). Although the collective data did indicate that 21S pre-rRNA can mature, the severely impaired growth of all of these mutants suggested it to be a rather poor substrate for 18S rRNA synthesis. The complete lack of cleavage of 32S pre-rRNA at site A_2 in the *rrp5Δ6* mutant, however, had no significant effect on the rate of growth of the cells, demonstrating that 21S pre-rRNA can be processed into mature 18S rRNA with an efficiency similar to that of the normal 20S precursor. In fact, the recent detection of low levels of 21S pre-rRNA even in wild-type cells (21) suggests that direct

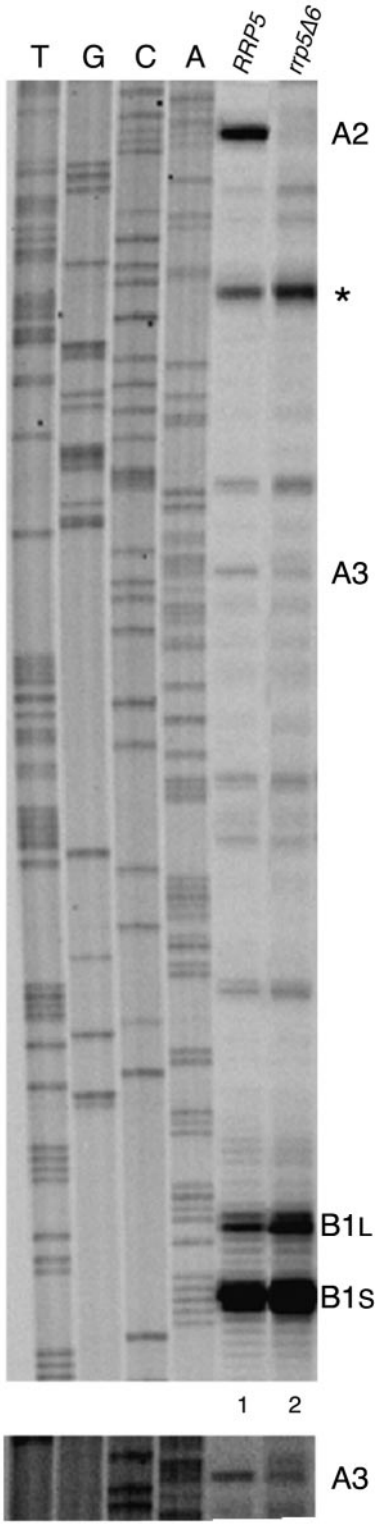

FIG. 5. Reverse transcription analysis of total RNA isolated from exponentially growing YJV163 (lane 1) or YJV207 (lane 2) cells expressing ProtA-Rrp5p or ProtA-Rrp5Δ6p, respectively, with probe 5 (Fig. 1A). The lower panel shows a longer exposure of the area of the same gel containing the stop at site A_3. The signal marked with an asterisk is an artificial stop always observed with these strains (17).

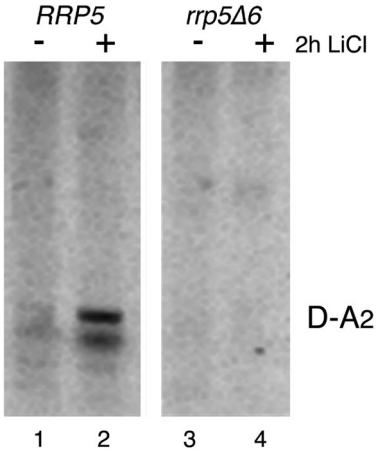

FIG. 6. Analysis of unstable low-molecular-weight RNA molecules. Total RNA was isolated from exponentially growing cells expressing ProtA-Rrp5p (lanes 1 and 2) or ProtA-Rrp5Δ6p (lanes 3 and 4) with or without the addition of LiCl to a final concentration of 0.2 M and further incubation for 2 h. RNA was separated on a denaturing 8% polyacrylamide gel and subjected to Northern analysis with probe 2 (Fig. 1A).

cleavage of 32S pre-rRNA at site A_3 may be used as a minor alternative pathway for pre-rRNA processing in yeast strains.

A comparison of the polysome profile of the *rrp5Δ6* mutant with that of its wild-type parent did not reveal any significant differences (data not shown), a finding which we take as further evidence that pre-rRNA processing via 21S and $27SA_3$ pre-rRNAs is a bona fide alternative pathway.

Maturation of 21S pre-rRNA. Regarding the manner in which 21S pre-rRNA matures, it should be noted that our data do not exclude the possibility that the absence of S1 motifs 10 to 12 disturbs the correct relative timing of the cleavages at sites A_2 and A_3 rather than inhibiting cleavage at site A_2. Thus, 21S pre-rRNA may still be a substrate for the endonuclease cutting at site A_2, resulting in the formation of the normal 20S precursor species. To assess the validity of this scenario, we performed Northern analysis with probe 2 of total RNA from exponentially growing cells expressing Rrp5Δ6p or wild-type Rrp5p, both before and after treatment of the cells with 0.2 M LiCl. In the absence of methionine, the addition of LiCl indirectly inhibits the 5′→3′ exonucleases Rat1p and Xrn1p (16), the latter of which is primarily responsible for the degradation of the D-A_2 fragment produced by the conversion of 20S pre-rRNA into mature 18S rRNA (40). The D-A_2 fragment was readily detectable in the RNA sample from wild-type cells treated with LiCl (Fig. 6, lane 2). In accordance with earlier results (46), we noted some heterogeneity in this fragment which was likely due to incomplete inhibition of the exonucleases. No D-A_2 fragment could be detected in either untreated or LiCl-treated *rrp5Δ6* cells (Fig. 6, lanes 3 and 4), suggesting that 21S pre-rRNA was not cleaved at site A_2. However, there also was no trace of a D-A_3 fragment, a finding which may indicate that the remaining portion of ITS1 is removed from 21S pre-rRNA by exonucleolytic digestion rather than endonucleolytic cleavage.

To determine whether cleavage at site D is required for the maturation of 21S pre-rRNA, we used a previously created

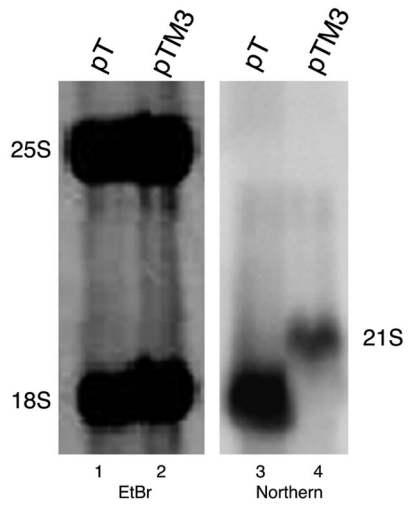

FIG. 7. Effect of mutation of site D on the maturation of 21S pre-rRNA. Strain YJV207 expressing Rrp5Δ6p was transformed with either plasmid pT, encoding a tagged, wild-type rDNA unit, or pTM3, encoding a tagged, mutant rDNA unit that carries a 4-nt substitution mutation across site D which inhibits cleavage at this site (46). Total RNA was isolated from both types of transformants (lanes 1 and 3 or lanes 2 and 4, respectively) and separated on an 8% polyacrylamide gel. The gel was stained with ethidium bromide (EtBr) to visualize total mature rRNA (lanes 1 and 2). Northern analysis was performed with a probe complementary to the tag in the 18S sequence (50) to detect plasmid-derived 21S pre-rRNA and 18S rRNA (lanes 3 and 4).

cis-acting substitution mutation of the 4-nt region spanning site D (mutation M3), which completely inhibits this cleavage (46). The *rrp5Δ6* strain was transformed with an rDNA plasmid (pTM3) that contains mutation M3 as well as a neutral tag in the 18S rRNA sequence (50), allowing discrimination between plasmid-derived and endogenous pre-rRNAs or rRNAs. Total RNA was isolated from the resulting strain as well as a control strain transformed with a similarly tagged plasmid lacking the M3 mutation (pT) and was subjected to Northern analysis with a probe complementary to the tag. Whereas *rrp5Δ6* cells transformed with either pTM3 or pT contained comparable levels of endogenous 18S and 25S rRNAs, as visualized by ethidium bromide staining (Fig. 7, lanes 1 and 2), tagged 18S rRNA was visible only in cells containing pT (lane 3). In cells transformed with pTM3, the plasmid-derived 21S pre-rRNA containing the mutant site D region completely failed to mature (Fig. 7, lane 4). These results demonstrate that cleavage at site D remains a crucial step in the conversion of the 21S precursor into mature 18S rRNA. However, the absence of the expected D-A_3 fragment in LiCl-treated *rrp5Δ6* cells (Fig. 6) indicates that the fragment is degraded in a manner different from that established for its normal D-A_2 counterpart. An obvious candidate for this degradation is the exosome, the major 3′→5′ exonuclease complex in eukaryotic cells, for which a role in the removal of other spacer fragments has already been established (3, 4, 33).

To investigate the possible involvement of the exosome in 21S pre-rRNA processing, we constructed an *rrp5Δ6* strain in which this exonuclease complex can be conditionally inactivated. Yeast strain YCA20 (Table 1) possesses a gene encoding the essential exosome component Rrp45p under the control of the repressible *GAL10* promoter. This strain was transformed with a linear construct containing the *ProtA*::*rrp5Δ6* gene as well as a *LEU2* marker. Genomic DNA was isolated from Leu^+ colonies, and the successful replacement of the wild-type *RRP5* gene with its *ProtA*::*rrp5Δ6* counterpart was confirmed by PCR. Positive strains were checked for the expression of ProtA-Rrp5Δ6p by Western blot analysis (data not shown). The resulting double-mutant strain YCA20-Δ6 (*GAL*::*rrp45 ProtA*::*rrp5Δ6*) and the wild-type control strain YCA20 (*GAL*::*rrp45 RRP5*) were grown in GRS medium and then shifted to glucose-based medium for 8 h, resulting in the depletion of Rrp45p and thus in the inactivation of the exosome (3). At 1 h before harvest, LiCl was added to portions of both GRS- and glucose-grown cultures to also block Rat1p and Xrn1p activities. Total RNA then was isolated from both LiCl-treated and untreated cultures and was subjected to Northern hybridization (Fig. 8).

In accordance with the data shown in Fig. 6, the D-A_2 fragment was detected by probe 2 in LiCl-treated wild-type YCA20 cells both before and after the shift to glucose (Fig. 8A, lanes 2 and 4), whereas YCA20-Δ6 cells completely lacked this fragment, irrespective of the growth conditions (lanes 5 to 8). However, when we depleted the exosome from the latter mutant and, at the same time, inhibited 5′→3′ exonuclease activity by LiCl treatment, probe 2 detected a fragment that migrated more slowly than the D-A_2 fragment (Fig. 8A, lane 8). The same fragment also hybridized with probe 3, located between sites A_2 and A_3 (Fig. 8B), but not with probe 4, located downstream from A_3 (Fig. 8C). Together with the estimated size of ~280 nt, these data indicate that the product in question corresponds to the D-A_3 fragment. We conclude that the aberrant 21S pre-rRNA is efficiently converted into 18S rRNA in the same manner as the normal 20S precursor. In contrast to the D-A_2 fragment, however, which is primarily degraded by 5′→3′ exonuclease digestion (16), the D-A_3 fragment is subject to efficient degradation from either end, since it can only be detected when the activity of the 5′→3′ exonucleases as well as that of the exosome is inhibited.

In this experiment, we also detected small amounts of products corresponding to A_2-E and A_3-E fragments (Fig. 8), which arose from a reversed order of ITS1 and ITS2 processing, respectively. These products were observed previously in wild-type and various mutant yeast strains (17).

DISCUSSION

Rrp5p occupies an exceptional position among the many *trans*-acting factors that participate in eukaryotic ribosome biogenesis, because contrary to the large majority of these factors, it is involved in the formation of both subunits. Previous mutational analysis indicated close agreement of this functional dichotomy of Rrp5p with its division into two distinct structural domains (Fig. 2). The C-terminal TPR domain was found to contain several elements essential for the early cleavages at sites A_0 to A_2 and thus the formation of the small subunit. The eight proximal N-terminal S1 motifs, on the other hand, appeared to be important for cleavage at site A_3 and the consequent predominant production of the short form of the large-subunit 5.8S rRNA. For the four distal S1 copies (9 to 12; amino acids 977 to 1354), no functional role was assigned,

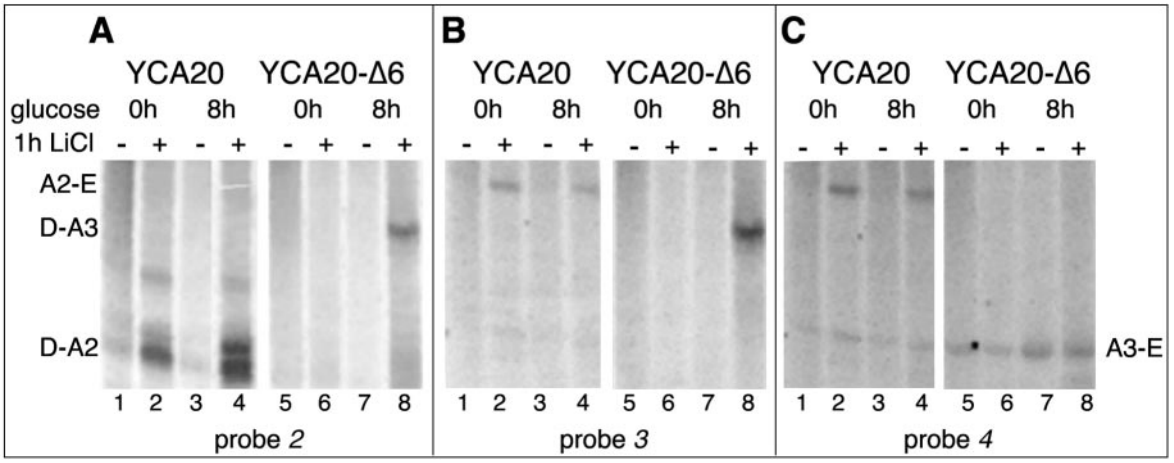

FIG. 8. Effect of inactivation of exonucleases on the accumulation of unstable, low-molecular-weight processing products. YCA20 cells (*GAL*::*rrp45 Prot*::*rrp5*) (lanes 1 to 4) and YCA20Δ6 cells (*GAL*::*rrp4 ProtA*::*rrp5Δ6*) (lanes 5 to 8) were harvested immediately before and 8 h after a shift from GRS- to glucose-based medium (odd-numbered lanes). Equal parts of the cultures were also treated with 0.2 M LiCl for 1 h before harvest (even-numbered lanes). RNA was separated on a denaturing 8% polyacrylamide gel, and Northern analysis was performed. Figure 1A shows the locations of the probes.

because their removal did not have any noticeable effect upon growth or the steady-state levels of the mature rRNA species (18). However, the results reported in this article necessitate a refinement of this view of structure-function relationships in Rrp5p. As is evident from both Northern and primer extension analyses (Fig. 5 and 6) of the *rrp-Δ6* mutant, the region containing S1 motifs 10 to 12 (amino acids 1132 to 1354) is essential for cleavage at site A_2, the first of the two processing steps that normally produce the mature 3′ end of 18S rRNA. Consequently, this portion of Rrp5p should be grouped functionally with the adjacent TPR domain, rather than the S1 domain, although it should be stressed that both A_0 and A_1 cleavages are not affected by deletion of S1 motifs 10 to 12. Interestingly, the revised partitioning of Rrp5p into functional domains conforms well to our finding that two contiguous fragments of the protein that abut at amino acid 1131 supply full Rrp5p functionality when expressed in *trans* (18). Individual deletion of S1 motif 9 (mutant *rrp5*Δ5) (18) had no effect on the levels of $27SA_2$ and 20S pre-rRNAs (H. R. Vos, unpublished data); therefore, this motif is the only one of the 12 that remains functionless.

The last S1 motif (12) of Rrp5p is separated from the first TPR motif by a region of about 100 amino acids (residues 1345 to 1456) that does not contain any identifiable structural motifs and that has not been individually subjected to mutational analysis (Fig. 2). However, Torchet and Hermann-Le Denmat have shown that yeast cells expressing a bipartite *RRP5* allele that encodes two noncontiguous fragments of the protein containing S1 motifs 1 to 9 (amino acids 1 to 1131; Rrp5N2) and TPR motifs 1 to 7 (amino acids 1457 to 1729; Rrp5C1) (Fig. 2) are viable, although they bypass processing at site A_2 and show a severe growth defect resulting from the underproduction of functional 40S subunits (43). Since, compared to the Rrp5Δ6 protein, the bipartite Rrp5N2-Rrp5C1 protein contains an extra deletion of amino acids 1345 to 1456, it seems likely that the impaired growth is due to the lack of the latter region. Interestingly, the growth defect can be partially suppressed by the overexpression of snR10 snoRNA (44). Since this overexpression enhances polysome formation without restoring cleavage at site A_2 or increasing the total amount of 40S subunits, it was suggested that snR10 overexpression improves 40S subunit assembly in the bipartite mutant, resulting in a higher percentage of functionally competent subunits. Thus, amino acids 1345 to 1456 of Rrp5p may constitute a further functional domain that is not involved in pre-rRNA processing but that assists in the correct assembly of 40S subunits.

Partial or complete bypassing of site A_2 and the consequent formation of 21S pre-rRNA were observed before upon mutation or depletion of several other *trans*-acting factors (6, 12, 42), as well as some small-subunit ribosomal proteins (41). However, the severe growth defects or even lethality as well as the significant reduction in 40S subunit formation invariably resulting from these mutations suggested that the 21S pre-rRNA is a considerably poorer substrate for 18S rRNA synthesis that its normal 20S counterpart. A similar conclusion was drawn for the bipartite *rrp5N2-rrp5C1* mutant discussed above (43). The recent detection of a small amount of 21S pre-rRNA in cells showing no growth defect (21) cannot be taken as evidence for its efficient conversion into 18S rRNA, since the large majority of 32S pre-rRNA in these cells is processed via the initial cleavage at site A_2. The *rrp5Δ6* mutant is the first strain that shows a complete lack of cleavage at site A_2 without detectable reductions in the amount of 18S rRNA and growth rate, demonstrating that cleavage at site A_2 can be bypassed without any negative consequences for the production of functional ribosomes. We therefore conclude that the alternative processing route involving the direct cleavage at site A_3 of 32S pre-rRNA into 21S and $27SA_3$ rather than 20S and $27SA_2$ pre-rRNAs is as effective as the normal one.

Maturation of 21S pre-rRNA proceeds via the same mechanism as that used for the conversion of the 20S precursor into 18S rRNA; i.e., it depends upon endonucleolytic cleavage at site D, the mature 3′ end of 18S rRNA. This conclusion follows from the complete inhibition of 18S rRNA production by the

M3 mutation (Fig. 7), which inhibits cleavage at site D (46), as well as the detection of a D-A_3 fragment in *rrp5Δ6* mutant cells (Fig. 8). Since cleavage at site D is a cytoplasmic event (36, 40, 49), the presence of the extra ITS1 sequence does not appear to hamper the nuclear export of 21S pre-rRNA, which is part of a 43S preribosome (43). However, whereas the D-A_2 fragment is degraded exclusively from the 5′ end (40), the D-A_3 fragment can be efficiently eliminated by digestion from the 5′ as well as the 3′ terminus, since this fragment can be detected only when both the exosome and the major 5′→3′ exonucleases are inhibited (Fig. 8). The reason for the different sensitivities of the two fragments to attack by the exosome remains to be established. However, 3′-shortened forms of 21S but not 20S pre-rRNAs were detected previously (2), indicating that the 3′ end of the former molecule is accessible to the exosome within the 43S preribosomal particle, whereas the 3′ end of 20S pre-rRNA is not. It is possible that the exosome, which is a processive enzyme, can be loaded onto the precursor before the cleavage at site D takes place.

In multicellular organisms, the existence of two alternative, efficient routes for pre-rRNA processing is a well-established fact, even to the extent that both can operate simultaneously in the same cell (23). Like ITS1 processing in the *rrp5Δ6* mutant, either pathway includes only a single internal cleavage within ITS1 (site 3), which is thought to be carried out by RNase MRP (47) but also to require U3, although this small nucleolar ribonucleoprotein plays only a supportive and not an essential role (11). On the other hand, generation of the mature 3′ end of 18S rRNA (site 2) in higher eukaryotes, unlike processing at yeast site D, is completely dependent upon U3 (23), as is cleavage at yeast site A_2. Thus, traits of yeast site A_2 recur in both site 2 and site 3 of higher eukaryotic ITS1, and the latter also may be similar to yeast site A_3. Since the data presented in this article demonstrate that, in terms of the production of functional ribosomes, cleavage at site A_2 is fully dispensable, the question arises as to why this cleavage still is part of the normal pre-rRNA processing pathway in yeast cells. A possible answer to this question may lie in the higher sensitivity of *rrp5Δ6* cells to antibiotics which, in a natural habitat, would be a disadvantage. Even though the ribosomes produced in the mutant cells are fully functional in the absence of antibiotics, shifting a portion of ITS1 from 66S to 43S preribosomes may have subtle effects on subunit assembly that increase the sensitivity of either the preribosomal particles or the completed subunits to these antibiotics. The hypersensitivity of *rrp5Δ6* cells to hygromycin B and cycloheximide indicates that the assembly of the large subunit may be affected in particular (7, 26, 35).

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